APPENDIX

VERSION OF SPECIFICATION SEQUENCE PARAGRAPHS WITH MARKINGS TO SHOW CHANGES MADE

A marked-up version of amended specification paragraphs showing changes made are provided below.

On page 13, line 3, after Ac-Pro-Gln-Ala-Tyr~Pro-Ile-Gln-Thr, please insert --(SEQ ID NO: 1)-- as follows:

Each compound tested in this study is a competitive inhibitor and is significantly more potent against HIV PR than FIV PR, as expected. However, the new inhibitors **1b-6b**, and **7a** showed a remarkable different pattern of inhibitory activities compared to their parent compounds **1a-5a**, ABT-538 and RO31-8959. Compounds **2a-4a**, containing no P3 moieties, exhibited marginal activities with IC_{50} value in the range of 2-300 μM against HIV PR and did not show any significant inhibition of FIV PR at 800 μM. Only the α-keto amide **5a** showed reasonable potency against HIV PR with a K_i value of 214 nM (Slee et al. J. Am. Chem. Soc. 1995, 117, 11867-11878). On the other hand, the modified inhibitors **2b-5b**, which contain a methyl group as P3 residue, displayed 120- to 1000- fold improved inhibitory activities against HIV PR and at least three orders of magnitude higher potency for FIV PR compared to their parent compounds. In particular, **5b** was found to be a slow binding inhibitor with K_i of 2.5 and 46 nM against HIV and FIV PR, respectively. This level of potency against FIV PR by the inhibitor with a molecular weight of only 649 was truly remarkable, considering the smallest efficient substate for the enzyme is an eight-residue peptide, Ac-Pro-Gln-Ala-Tyr~Pro-Ile-Gln-Thr (SEQ ID NO: 1) (Schnlzer et

al. Virology 1996, 224, 268-275).

On page 25, line 26, after Ac-Gln-Ala-Tyr~Pro-Ile-Gln, please insert --(SEQ ID NO: 2)-- and on page 26, line 1, after Ac-Gln-Ala-Tyr~Pro-Ile-Gln please insert --(SEQ ID NO: 1)--. Also, on page 26, line 1 please amend the specification to correct a typographical error as follows. Support for the correction to the typographical error can be found on page 26, line 18.

All the C_2-symmetric diols tested in this study showed very high potency against HIV PR, and their K_i's ranged between 1.1 and 2.6 nM. Considering experimental error, there was no significant difference in the overall efficacy of these diols in inhibition of HIV PR. In part, this reflects the low restriction of amino acid residues at the S3 and S3′ subsites of HIV PR. In addition, the Cbz groups of the reference inhibitor **1000**, which does not contain P3 and P3′ residues, could be positioned tightly at the S3 and S3′ subsites of HIV PR to make compound **1000** an effective inhibitor. However inhibition of FIV PR by inhibitors **1000-1400** showed a remarkably different pattern. First, the inhibitory activity of the reference compound **1000** was decreased by almost $1.7x10_4$ fold compared to its K_i for HIV PR. This striking activity loss observed for **1000** was recovered by extending the backbone of the inhibitor using Gly as P3 and P3′ residues, with the K_i of **1100** being 110 fold lower than **1000**. This preference of the extended inhibitor backbone found in FIV PR is also supported by the observation that HIV PR will cleave a six residue peptide substrate, Ac-Gln-Ala-Tyr~Pro-Ile-Gln (SEQ ID NO: 2), whereas the smallest FIV PR substrate is an eight residue

peptide, Ac-Gln-Ala-Tyr~Pro-Ile-Gln (SEQ ID NO: 1). The best residue for S3 and S3[¢]′ binding was Ala. In fact, inhibitor **1200** (K_i = 41 nM) is the most potent inhibitor of FIV PR known to date. The inhibitory activity of **1200** against FIV PR was reduced by increasing the size of the side chain of the P3 and P3′ residues, with the K_i of **1300** 4-fold higher than **1200**. Furthermore, the diol **1400** showed 45- and 170-fold lower potency compared to **1100** and **1200**, respectively, and this result suggests that the benzyl side chain of P3 and P3′ may cause unfavorable interaction with FIV PR or the neighboring P1 and P1′ side chains. This severe restriction of P3 and P3′ moieties in FIV PR partly explains the total loss of potency against FIV PR by HIV PR inhibitor Ro 31-8959, since it contains bulky aromatic group at the P3 position.

At page 33, line 10, after Abz-Thr-Ile-Nle-Phe-(*p*-NO_2)-Gln-Arg-NH_2, please insert --(SEQ ID NO: 3)-- as follows:

For determination of IC_{50} values for HIV protease, a backbone engineered HIV-1 protease, prepared by total chemical synthesis (Kent et al. Science 1992, 256, 221) 450 nM final concentration was added to a solution (152 µL final volume) containing inhibitor, 28 µM fluorogenic peptide substrate (sequence Abz-Thr-Ile-Nle-Phe-(*p*-NO_2)-Gln-Arg-NH_2 (SEQ ID NO: 3) (Toth et al., Int. J. Peptide Res. 1990, 36, 544)) and 1.8% dimethylsulfoxide in assay buffer: 100nM MES buffer containing 0.5 mg/mL BSA (Bovine Serum Album, fatty acid, nuclease and protease free - to stabilize enzyme) at pH 5.5. The solution was mixed and incubated over 5 minutes during which time the rate of substrate cleavage was monitored by continuously recording the

change in fluorescence of the assay solution. An excitation filter of 325 nm, and an emission filter of 420 nm were used. This data was converted into μM substrate cleaved per minute, using a predetermined standard calibration curve of change in fluorescence against concentration of substrate cleaved.

At page 34, lines 5-7, after Gly-Lys-Glu-Glu-Gly-Pro-Pro-Gln-Ala-Tyr~Pro-Ile-Gln-Thr-Val-Asn-Gly, please insert --(SEQ ID NO: 4)-- as follows:

For determination of K_i and IC_{50} for FIV protease, 0.125 μg of the enzyme was added to a solution (100μL final volume) containing inhibitor, 560 μM peptide substrate (sequence Gly-Lys-Glu-Glu-Gly-Pro-Pro-Gln-Ala-Tyr~Pro-Ile-Gln-Thr-Val-Asn-Gly (SEQ ID NO: 4)) and 2% dimethyl sulfoxide in a 1:3 mixture of assay buffer (as above) and 4M $NaCl_{aq.}$ solution. The solution was mixed and incubated for 10 minutes at 37° C and the reaction quenched by addition of 8M guanidine HC1 solution containing 0.2 M sodium acetate at pH 4.2 (100 μL). The cleavage products and substrate were separated by reverse phase HPLC. Absorbance was measured at 215 nm, peak areas were determined and percent conversion to product was calculated using relative peak areas. The data were plotted as 1/V (V = rate substrate is cleaved in nmol/min) against inhibitor concentration and the $-K_i$ determined as the point at which the resulting line intersects with $1/V_{max}$ (V_{max} = 6.85 nmol/min). IC_{50} was determined as the inhibitor concentration at 50% inhibition. V_{max} (6.85 ± 0.7 nmol min^{-1}) and K_m (707 ±70μM) for FIV protease were determined from a plot of 1/V (V = rate in nmol/min) against 1/[S] ([S] = substrate concentration in nmol). The data used was generated similarly to that for K_i with the following modifications. The substrate

concentrations used were 560, 448, 336, 224, 111 and 56 µM, in the absence of inhibitor.

At page 59, line 27 - page 60 line 1, after (Abz)-Thr-Ile-Nle~Phe-(*p*-NO_2)-Gln-Arg-NH_2, please insert --(SEQ ID NO: 3)-- as follows:

For HIV PR, the K_M and V_{max} values for the fluorogenic peptide substrate 2-aminobenzoyl (Abz)-Thr-Ile-Nle~Phe-(*p*-NO_2)-Gln-Arg-NH_2 (SEQ ID NO: 3) Toth et al Int. J. Peptide Res. 36, 544-550 were determined by measuring the initial rate of hydrolysis at different substrate concentrations (5.0, 7.5, 10, 20, 35, 50, 100, and 200 µM) by monitoring the change in fluorescence at an excitation wavelength of 325 nm and an emission wavelength of 420 nm, and fitting the obtained data to the Michaelis-Menten equation using the Grafit program (version 3.0, Erithacus Software Ltd., UK). Assays were run in 0.1 M MES buffer, containing 5% (*v/v*) glycerol, and 5% (*v/v*) DMSO (200 µl final volume). The enzyme concentration (30 µg/ml) which gave ideal progress curve was used for assays, but the dimeric active HIV PR concentration was not accurately determined. The K_i for each inhibitor of HIV PR was determined by obtaining the progress curve with the inhibitor (2.0 - 9.0 nM) at different substrate concentrations (7.5, 10, 20, 35, and 50 mM), under the same reaction conditions as above. The curve fit the data was determined, and the subsequent K_i was derived using the Grafit program.

At page 60, lines 22-23, after Arg-Ala-Leu-Thr-Lys(Abz)-Val-Gln~nPhe-Val-Gln-Ser-Lys-Gly-Arg, please insert --(SEQ ID NO: 5)-- as follows:

For FIV PRs, the kinetic data were determined under the similar reaction conditions as for HIV PR. The K_M and V_{max} for the fluorogenic substrate Arg-Ala-Leu-Thr-Lys(Abz)-Val-Gln~nPhe-Val-Gln-Ser-Lys-Gly-Arg (SEQ ID NO: 5) were determined by monitoring the change in fluorescence at an excitation filter of 325 nm and an emission filter of 410 nm with the Grafit program under the following reaction conditions: substrate concentration (6.0, 10, 20, 35, 50, 100, and 200 µM), 0.1 M NaH_2PO_4 buffer containing 0.1 M Na citrate, 0.2 M NaCl, 1.0 mM DTT, 5% (*v/v*) glycerol, 5% (*v/v*) DMSO and 7.5 µg/ml (FIV(3X) and FIV(V59I)) or 2.5 µg/ml (FIV(Q99V)) of the enzyme. The K_i for each inhibitor of FIV PRs was also determined by obtaining the progress curve with the inhibitor (50 nM - 20 µM) at different substrate concentrations (10, 20, 35, and 50 µM).

At page 61, line 27, after 5'ATCTCTCCCCAATAATGGTACTATTAATGAGTTATCTTCT AAGAC3' please insert --(SEQ ID NO: 6)--, on page 62, line 3, after 5'ACTATTGGACATATGGCATATAATAAAGTAGG TACTACTAC3' please insert --(SEQ ID NO: 7)-- and, on page 62, line 10, after 5'ATCAGAAAGCTTTTACATTACTAACCTGATATTAAATTT3' please insert --(SEQ ID NO: 8)-- as follows :

(FIV(Q99V). The feline immunodeficiency virus 34TF10 infectious molecular clone (FIV-34TF10) was used as the template in a polymerase chain reaction (PCR) using a negative strand primer (5'ATCTCTCCCCAATAATGGTACTATTAATGAGTTATCTTCT AAGAC3' (SEQ ID NO: 6); complementary to nt's 2252-2297) which mutated the FIV PR Gln 99 codon to Val and the positive strand primer (5'ACTATTGGACATATGGCATATAATAAAGTAGG TACTACTAC3' (SEQ ID NO: 7); nt's 1964-2005) which, when incorporated into the PCR product,

added an initiation Met and Ala codon to the determined 5' Tyr codon of the FIV/PR open reading frame (ORF; 19) as well as a 5' Nde I restriction site. The ~300 bp PCR product was purified and used in a second PCR with the same template and with a negative strand primer (5'ATCAGAAAGCTTTTACATTACTAACCTGATATTAAATTT3' (SEQ ID NO: 8); complementary to nt's 2306-2345) which added a stop codon after the determined C-terminal Met codon of the PR ORF in addition to a 3' Hind III restriction site, to facilitate cloning. The resulting PCR product was digested with Nde I and Hind III and ligated into pT7-7 (^{35}), which had been digested with Nde I and Hind III, to give FIV(Q99V).

At page 62, line 19, after 5'GGAAGGCAAAATATGATTGGAATTGGAGGAGGAAAGAGAGGAACA3' please insert --(SEQ ID NO: 9)-- as follows:

FIV(V59I). FIV-34TF10 was the template in a PCR reaction with the positive strand primer (5'GGAAGGCAAAATATGATTGGAATTGGAGGAGGAAAGAGAGGAACA3' (SEQ ID NO: 9); nt's 2135-2178) which mutates the FIV PR Val codon 59 to Ile, and the second negative strand primer used for FIV(Q99V). The ~200 bp PCR product was purified and used in a second PCR with the same template and the positive strand primer used for FIV(Q99V). The resulting PCR product was digested with Nde I and Hind III and ligated into pT7-7, which had been digested with Nde I and Hind III, to give FIV(V59I).